Direct cell-cell interaction of cardiomyocytes is key for bone marrow stromal cells to go into cardiac lineage in vitro

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Objectives: Cardiac environmental factors are thought to be powerful inducers in cardiomyogenic differentiation. In this study we simulated the cardiac environment using coculture and evaluated the cardiomyogenic differentiation in bone marrow stromal cells.

Methods: In group 1 only bone marrow stromal cells derived from transgenic mice expressing green fluorescent protein (GFP-BMCs) were cultured ($n = 5$). In group 2 cardiomyocytes from neonatal rats were grown on inserts, which we applied to culture dishes seeded with GFP-BMCs ($n = 5$). In group 3 GFP-BMCs were cocultured with cardiomyocytes on the same dishes ($n = 5$). We cultured these cells for 7 days and evaluated the synchronous contraction and the cardiomyogenic differentiation of GFP-BMCs by means of immunostaining.

Results: In groups 1 and 2 GFP-BMCs protein did not show any myogenic phenotypes for 7 days. In contrast, in group 3 some GFP-BMCs were incorporated in parallel with cardiomyocytes and revealed myotube-like formation on day 1. On day 2, some GFP-BMCs started to contract synchronously with cardiomyocytes. Myosin heavy chain-positive GFP-BMCs were recognized in $2.49\% \pm 0.87\%$ of the total GFP-BMCs on day 5 ($P < .0001$). Cardiac-specific troponin I-positive GFP-BMCs were in $1.86\% \pm 0.53\%$ of the total cells on day 5 ($P < .0001$). Atrial natriuretic peptide was also seen in GFP-BMCs, and connexin 43 was detected between GFP-BMCs and cardiomyocytes.

Conclusions: Direct cell-cell interaction with cardiomyocytes was important for bone marrow stromal cells to differentiate into cardiomyocytes. This coculture was useful for simulating the cardiac environment in vitro for the research of cell transplantation in the heart.

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Bone marrow cell may be a candidate for cell-based therapy for regenerating many kinds of tissue such as liver, neuron, fat, and tendon,¹ and we previously reported that transplantation of bone marrow stromal cells (BMCs) induced myogenesis and angiogenesis in damaged hearts and improved impaired function.^{2,3}

A part of the transplanted bone marrow cells differentiated into cardiomyocytes without any artificial manipulation in vivo.^{2,4-6} Even xenogeneic stem cells went to site-specific differentiation in the body of another species.^{7,8} These data suggested that environmental factors were natural inducers of differentiation. The heart might have the capacity to regenerate itself when it is damaged.⁹ The effects are very difficult to investigate, however, because of their in vivo nature. We hypothesized that direct attachment between BMCs and cardiomyocytes was one of the environmental inducers.

In this study we simulated the cardiac environment with coculture composed of green fluorescent protein mouse-BMCs (GFP-BMCs) and rat cardiomyocytes and

Figure 1. Experimental culture system with rat cardiomyocytes (*CM*) and GFP-BMCs.

Figure 2. The morphology of GFP-BMCs in passage 2 (A and B) and rat cardiomyocytes (*CM*; C and D) in vitro. A, These cells were spindle, oval, wedge, or sheet shaped. B, All cells expressed green under fluorescent microscopy. (Original magnification 200 $\times$.) D, None of the cells were visible under fluorescent microscopy. (Original magnification 200 $\times$.)

report, for the first time to our knowledge, that BMCs differentiate into cardiomyocytes in a coculture and cell-cell attachment is one of the environmental factors of differentiation.

Materials and Methods

Subjects

Animals were studied on the basis of the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Labora-

Figure 3. GFP-BMCs with rat cardiomyocytes (CM) on day 2 after coculture in group 3. GFP-BMCs were spindle shaped, attached to cardiomyocytes, and contracted synchronously with cardiomyocytes. (Original magnification 200 $\times$.)

Figure 4. The proliferation of GFP-BMCs in groups 1 (solid line) and 3 (dotted line) is shown. There was no difference between groups 1 and 3 (NS).

tory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996, and approved by the Institutional Animal Care and Use Committee at the National Cardiovascular Center Research Institute. Pregnant Sprague-Dawley rats were purchased from a licensed vendor. Transgenic mice expressing green fluorescent protein (C57BL/6Tg14[act-EGF-P]OsY01: GFP mouse) were kindly provided by Dr M. Okabe.¹⁰ Animals were housed in an air-conditioned room, with free access to food and water at all times.

BMCs From GFP Mice

A GFP mouse was anesthetized with diethylethanol. After achievement of general anesthesia, the femora and tibiae were collected.^{4,11} After removing connective tissue around the bone, both ends of the bone were cut. Bone marrow plugs were flushed with

a 27-gauge needle and a syringe filled with complete medium (Iscove modified Dulbecco medium with 10% fetal bovine serum, 100 U/mL penicillin G, and 100 μ g/mL streptomycin). Cells were introduced into 100-mm dishes and incubated at 37°C in 5% carbon dioxide and 95% air. Three days later, the medium was changed, and the nonadherent cells were discarded. Medium was completely replaced every 3 days. Passage was done when confluency exceeded 70%. BMCs in passages 2 or 3 were used in this study. We operationally called these cells stromal cells.

Neonatal Rat Cardiomyocytes

Cardiomyocytes were isolated from 1-day-old newborn Sprague-Dawley rats.¹² In brief, neonatal rats were anesthetized with diethylethanol and killed by means of decapitation, and their hearts were rapidly removed and placed into dishes on ice. After the atria

Figure 5. Myogenic differentiation of GFP-BMCs. Cells were stained with a first antibody against MHC. A was photographed during the contrast phase. B and C were photographed during the fluorescent phase (B, green, excitation at 515-540 nm; C, red, excitation at 574-640 nm). D was the double-labeled cell superposition of B and C. Positive cells showed a cross-striated pattern. Combined green and red fluorescence represented myogenic cells derived from BMCs. (Original magnification 400 $\times$.)

and the great vessels were discarded, hearts were minced into 1-mm³ pieces with fine scissors, transferred to a sterile tube, and washed once in cold phosphate-buffered saline solution (PBS; NaCl, 136.9 mmol/L; KCl, 2.7 mmol/L; Na₂HPO₄, 8.1 mmol/L; and KH₂PO₄, 1.5 mmol/L [pH 7.3]) to remove any blood and clots. The minced tissue was digested in a PBS solution supplemented with 0.5% trypsin, 0.1% collagenase, and 0.02% glucose for 2 minutes at 37°C. The cell suspension was transferred into a tube containing 20 mL of complete medium and centrifuged at 1000 rpm for 5 minutes. The cell pellet was resuspended in complete medium and plated on 35-mm dishes (Falcon; Becton, Dickinson and Company, Franklin Lakes, NJ) at a density of $1.25 \times 10^4/\text{cm}^2$ and cultured at 37°C in 5% carbon dioxide and 95% air.

Experimental Culture Systems

GFP-BMCs and cardiomyocytes were prepared as described above, and 1×10^5 cells/dish were plated as follows (Figure 1). In group 1 only GFP-BMCs were plated on 35-mm dishes (Falcon) as

control specimens (n = 5). In group 2 cardiomyocytes were plated onto cell culture inserts (Falcon), which we applied to 35-mm dishes seeded with GFP-BMCs 2 days later (n = 5). In group 3, cardiomyocytes were plated on 35-mm dishes, followed by additional plating of GFP-BMCs 2 days later to make up a coculture (n = 5). They were incubated at 37°C in 5% carbon dioxide and 95% air until further processing. All the dishes were then evaluated for 1 week with a fluorescent microscope (Nikon TE300, Nihon Kogaku, Tokyo, Japan) equipped with a heated plate (37°C), a digital video camera, and a confocal microscope (Olympus Fluoview, Tokyo, Japan).

Immunohistochemistry

The cultured cells were immunohistochemically stained. In brief, the cells were washed with PBS and fixed with 4% paraformaldehyde for 5 minutes at room temperature, whereas the dishes for staining against anticonnexin 43 were fixed for 10 minutes at 4°C. A mouse monoclonal antibody against myosin heavy chain

Figure 6. The percentage of MHC-positive BMCs from day 0 through day 5 in group 3. The *bar* represents mean and SE. MHC-positive GFP-BMCs appeared from day 1. There was a significant difference among days in group 3 ($P < .0001$). As the days passed, the expression of MHC-slow significantly increased to 2.5% on day 5.

Figure 7. ANP-positive BMCs. Cells were stained with a first antibody against ANP. The phases (A-D) were the same microscopic conditions seen in Figure 5. (Original magnification 400 $\times$.)

Figure 8. The percentage of ANP-positive BMCs in group 3. The *bar* represents mean and SE. Positive cells appeared on days 2, 3, and 4. Among days, the difference in the percentages were recognized as significant ($P = .039$).

Figure 9. Connexin 43-positive BMCs. Cells were stained with a first antibody against connexin 43. Phases A and B were the same microscopic conditions as in Figure 5. Connexin 43 was detected at the margin of BMCs in phase C. (Original magnification 600 $\times$.)

Figure 10. TnI-positive BMCs. Cells were stained with a first antibody against TnI. The phases (A-D) were the same microscopic conditions as in Figure 5. Some BMCs stained positively and showed myofibrils lengthwise along the cell. The expression of TnI corresponded to green fluorescence derived from BMCs. (Original magnification 400 $\times$.)

(MHC)-slow (Sigma, St Louis, Mo) diluted 1:1000 was used to evaluate the differentiation of striated muscle. A rabbit monoclonal antibody against atrial natriuretic peptide (ANP; Protos Biotech Corp, New York, NY) diluted 1:1000 was used to determine the cardiac-specific expression. Connexin 43 was detected by using a rabbit polyclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, Calif) diluted 1:1000 and a mouse monoclonal antibody against cardiac-specific troponin I (TnI; Hytest, 4C2, Euro City, Finland) diluted 1:200 to detect mature cardiomyocytes. The dishes were incubated with the first antibodies at 4°C overnight. The culture dishes were washed with PBS 3 times to remove unbound antibodies. The primary antibodies anti-MHC-slow and anti-TnI were detected with a goat anti-mouse IgG antibody (Alexa Fluor 568, Molecular Probes, Wako, Osaka, Japan), and anti-ANP and anti-connexin 43 were detected with a goat anti-rabbit IgG antibody (Alexa fluor 568, Molecular Probes, Wako, Osaka, Japan). After incubation, the culture dishes were rinsed with PBS. The cells were then evaluated and photographed with a Fluoview FV300 confocal laser scanning microscope equipped with a z-stepping system (Olympus, Tokyo, Japan).

Quantitative Analysis

The percentage of positively stained cells was determined by using a fluorescent microscope, and the structure of the differentiated GFP-BMCs was observed in detail by means of confocal microscopy. In briefly, the total cell number was counted in the bright field. GFP-BMCs were detected with a band beam splitter for simultaneous excitation at 515 to 540 nm and counted. Alexa dye, which conjugated the cells, was visualized with a band beam splitter for simultaneous excitation at 574 to 640 nm. The percentage of positively stained cells was calculated in 4 randomly selected fields of 5 culture dishes from the initial plating (day 0) through the seventh day (day 7).

Statistical Analysis

Statistical analysis was performed with StatView 5.0 software (SAS Institute, Inc, Cary, NC). All values are expressed as means $\pm$ SE. Comparison of the growth rate between 2 distinct groups was analyzed by using the Mann-Whitney *U* test. Comparison of the data among days in each group was performed with the Kruskal-Wallis test.

Figure 11. The percentage of cardiac-specific TnI-positive BMCs in group 3. The *bar* represents mean and SE. Positive cells appeared on day 4 and increased to 1.9% on day 5. The differences of the positive BMCs were recognized to be significant among days ($P < .0001$).

Results

Morphologic Changes of GFP-BMCs and Cardiomyocytes

Although GFP-BMCs were cultured and passaged, nonadherent cells were eliminated, and spindle-shaped cells formed colonies and proliferated rapidly. All of the GFP-BMCs but red blood cells expressed bright green fluorescence (Figure 2). Contracting cardiomyocytes were identified on day 1. Contracting frequency per minute of beating cardiomyocytes was 60 to 70. On day 2, a few neonatal cardiomyocytes connected each other, and the beating rate was 70 to 80 per minute. None of the cardiomyocytes was visible under the fluorescent condition (Figure 2). In group 1 the shapes of these cells varied (ie, spindle, oval, wedge, or sheet), and they did not show any contraction. In group 2 GFP-BMCs did not contract. The shape and proliferation of GFP-BMCs were not different from those in group 1. In group 3, however, on day 1, part of the spindle-shaped GFP-BMCs attached in parallel to the colony of contracting cardiomyocytes ($12.5\% \pm 1.8\%$), whereas flattened GFP-BMCs covered the cardiomyocyte layer at random. On day 2, we found that GFP-BMCs attached to nonfluorescent contracting cells (cardiomyocytes) started to contract synchronously with cardiomyocytes ($5.6\% \pm 2.3\%$, Figure 3). The beating rate was almost 60 to 80 per minute. On day 5, GFP-BMCs began forming colonies and maintained synchronous contraction ($15.6\% \pm 4.2\%$). As time passed, the contracting cells communicated, and almost all the fields contracted synchronously. The proliferation of the GFP-BMCs between groups 1 and 3 was not different in this study (Figure 4). After day 6, the cultured cells were peeled off, and we could not evaluate them immunocytologically.

Phenotypic Changes of GFP-BMCs

In groups 1 and 2 GFP-BMCs did not express any type of myogenic or gap junction proteins. In contrast, in group 3 GFP-BMCs started to express MHC at $0.14\% \pm 0.09\%$ of the total GFP-BMCs on day 1. The MHC-positive BMCs increased day by day and were recognized at $2.49\% \pm 0.87\%$ on day 5 (Figures 5 and 6). The double-labeled cells indicated that striated muscles originated from GFP-BMCs, and almost all of these cells had dinuclei. The ANP-positive BMCs were detected mainly on days 2 and 3 ($0.78\% \pm 0.56\%$, Figures 7 and 8). As the days passed, the ANP-positive BMCs decreased on day 4 and disappeared on day 5. Connexin 43 was identified between GFP-BMCs and unlabeled cardiomyocytes from day 2 through day 5 (Figure 9). The cardiac-specific TnI-positive BMCs appeared at $1.11\% \pm 0.42\%$ on day 4 and increased to $1.86\% \pm 0.53\%$ on day 5 (Figures 10 and 11). The results are summarized in Table 1. Groups 1 and 2 did not show any myogenic differentiation of GFP-BMCs. In contrast, GFP-BMCs in group 3 expressed MHC first, followed by the expression of connexin 43 and ANP. Finally, GFP-BMCs expressed TnI. Some GFP-BMCs stained positive against myogenic proteins attached directly to cardiomyocytes and some attached to cardiomyocytes through nonmyogenic cells.

Discussion

The evidence of cardiomyogenic differentiation of bone marrow cells *in vivo* suggests the existence of environmental factors.^{2,4-6} However, these factors are not well known because they are *in vivo* phenomena, making investigation difficult. Possible factors might include cell-cell interaction,

Table 1. The trend of time-dependent expression of proteins

		0	1	2	3	4	5
Group 1		—	—	—	—	—	—
Group 2		—	—	—	—	—	—
Group 3	MHC	—	+	+	+	+	+
	connexin43	—	—	+	+	+	+
	ANP	—	—	+	+	+	—
	TnI	—	—	—	—	+	+

MHC: Myosin heavy chain slow. ANP: Atrial natriuretic peptide. TnI: Troponin I. +: some cells expressed protein. —: negative.

electrical and mechanical stimulation, and unknown growth factors.

We hypothesized the cell-cell interaction was a cardiogenic inducer for stem cells and set up a coculture to simulate the *in vivo* phenomena by using GFP-BMCs and cardiomyocytes, which have 2 advantages. First, GFP-BMCs are visible because the cells are alive. Contraction is a typical characteristic of myogenic cells, which is only seen in living cells. We can see the interaction between GFP-positive cells and GFP-negative cells dynamically. Second, GFP-BMCs facilitated 100% labeling efficiency, which enabled us to differentiate GFP-BMCs from cardiomyocytes. In other words it was possible to perform quantitative analysis of GFP-BMCs without false-negative and false-positive contamination. The cultured GFP-BMCs maintained green fluorescence strongly for at least 8 weeks. GFP-BMCs proliferated as C57 mouse-derived BMCs.

This study also has a characteristic: the simulation of xenogeneic cell transplantation from mice to rats *in vitro*. Even if xenogeneic cell transplantation has several issues, it might provide commercial availability in the future if immunologic problems are solved.⁷

Reinecke and colleagues¹³ reported that some skeletal myoblasts contracted synchronously with adjacent cardiomyocytes *in vitro*. However, skeletal myoblasts did not differentiate into cardiomyocytes. Makino and associates¹⁴ and ourselves² reported that BMCs were induced into cardiomyogenic cells with chemicals. In contrast, in this study we did not use any chemicals and only cocultured with cardiomyocytes. We showed here that multinuclei GFP-

BMCs differentiated into cardiomyogenic cells. GFP-BMCs started to contract synchronously with cardiomyocytes. Isoproterenol (25 nmol/L) increased the heart rate of the GFP-BMCs and the cardiomyocytes from 80 to 100 per minute (unpublished data). This mechanism could also happen *in vivo*.^{4,7}

There are some possible reasons why groups 1 and 2 did not show the cardiac differentiation of GFP-BMCs. Although BMCs have the capacity for cardiac differentiation, they might need some triggers, such as 5-azacytidine.^{2,14} Furthermore, unknown soluble inducers might not exist or might exist only at low concentrations. We regarded the direct attachment with cardiomyocytes as one of the important triggers for the cardiogenic differentiation of GFP-BMCs.

Our results indicated that GFP-BMCs cocultured with cardiomyocytes expressed myogenic protein as the first step, gap junction protein and ANP as the second step, and TnI as the final step. In contrast to increasing MHC and TnI values, ANP vanished on day 5. ANP is important for proliferation in embryonal cardiac development.¹⁵ Cardiomyogenic (CMG) cells from bone marrow stroma also expressed ANP.¹⁶ The myogenic cells might have lost ANP as a result of ventricular phenotypic change.

Although we observed the differentiation of GFP-BMCs, the percentage of differentiated cells was low. We considered the possible reasons. We did not purify specific cell types, such as CD34, in this study. Therefore, cultured cells were heterogeneously populated, and only a very small percentage of the BMCs were pluripotent stem cells, whereas most others were lineage-destined progenitor cells.

In this study we evaluated this coculture for only 1 week because the cultured cells were detached from the bottom of dishes as a result of overconfluency. Given the time-dependent increase of MHC- and TnI-positive cells, the percentage of cardiomyogenic cells from GFP-BMCs might increase if a longer culture is possible. We simulated BMC transplantation into the normal myocardium in this study. Stem cells are thought to be subtle in the normal tissue. On the other hand, injury, including ischemia, might trigger these cells to be active.

Cell fusion was suggested as an explanation for stem cell plasticity.^{17,18} In contrast, another group¹⁹ was against the fusion theory because single euploid multipotent adult progenitor cells differentiated into cells of 3 germ layers *in vitro*. They showed a high frequency of chimerism in comparison with the results of the previous study.¹⁷ Some *in vivo* studies have reported a robust (30%-50%) level of transdifferentiation.²⁰ Although we cannot exclude the possibility of cell fusion, our conversion rate (2.5%) was much higher than the frequency of spontaneous fusion (2-11 clones out of 10⁶ BMCs; 0.0002%-0.0011%).¹⁷ The mech-

anism of differentiation of stem cells should be investigated more deeply in future studies.

We used neonatal cardiomyocytes because we wanted to see contractions not seen in adult cardiomyocytes in vitro. The combination of adult cardiomyocytes and GFP-BMCs might be evaluated later.

The present study provides the first demonstration, to our knowledge, of the cardiomyogenic differentiation of BMCs without any chemicals in vitro. Using this coculture, we might be able to identify specific substances regulating cardiac development in the future.

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Discussion

Dr Frank W. Sellke (*Boston, Mass*). How do you know that it is due to direct cell-to-cell contact or interaction and that there is not some substance secreted that causes this effect?

Dr Tomita. Of course, from only the observation under the microscope, we do not know about that in detail. Some unknown soluble factors might go through gap junctions, and we speculated another mechanism of induction. We saw some TnI-positive cells derived from GFP-BMCs attached to GFP-negative cells, which were TnI negative. This observation suggested that some BMCs differentiated to cardiomyocytes by means of mechanical stretching. Therefore, there are several inducers in this system.

Dr Henry M. Spotnitz (*New York, NY*). What do you think the mediators are of this effect that are passing through the gap junctions?

Dr Tomita. Thus far I have no concrete evidence.

Dr Spotnitz. You are sure that these cells are being transformed and that they are not really myocytes?

Dr Tomita. Do you mean that the phenomenon is due to fusion?

Dr Spotnitz. Yes.

Dr Tomita. There were landmark articles regarding fusion between embryonic stem cells and BMCs published in the journal *Nature* in April. They include a warning that reported differentiation might be due to fusion. But in this study we just cultured cardiomyocytes and BMCs and not embryonic stem cells. Of course there are some possibilities, but embryonic stem cells are very energetic and immature. They are easy to communicate, and in the in vivo situation we put BMCs in the adult heart. They are not embryonic stem cells. Therefore, it is a different story.

Dr Marcio Scorsin (*Curitiba, Brazil*). I have some doubts concerning the fate of transplanted BMCs into a myocardial infarction scar. It is widely accepted that those cells might have a milieu-dependent differentiation (becoming cardiomyocytes) in normal myocardium. However, if you inject those cells into a myocardial scar, according to some studies, they would produce angiogenesis and differentiate into fibroblasts instead of cardiomyocytes. My question is whether you think that it is important to differentiate BMCs before transplantation.

Dr Tomita. For the in vivo study, it is not necessary to convert all BMCs into cardiomyocytes. For example, if you put BMCs into the scar, they might go in like myofibroblasts, but the myofibroblasts are also important to prevent extension of the scar. I saw some TnI-positive cells from transplanted BMCs in the scar tissue in the previous study. I agree to the hypothesis that fibroblasts are strong inducers for BMCs to transform to fibroblasts.